SULFUR MUSTARD-INDUCED INCREASE IN INTRACELLULAR CALCIUM: A MECHANISM OF MUSTARD TOXICITY

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ABSTRACT

The effect of sulfur mustard (SM, bis-(2-chloroethyl) sulfide) on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was studied in vitro using the clonal mouse neuroblastoma-rat glioma hybrid NG108-15 and primary normal human epidermal keratinocyte (NHEK) cell culture models. SM depletes cellular glutathione (GSH) and thus may inhibit GSH-dependent Ca^{2+}-ATPase (Ca^{2+} pump), leading to a high $[Ca^{2+}]_i$ and consequent cellular toxicity. Following 0.3 mM SM exposure, GSH levels decreased 20-34% between 1-6 hr in NG108-15 cells. SM increased $[Ca^{2+}]_i$, measured using the Ca^{2+}-specific fluorescent probe Fluo-3 AM, in both NG108-15 cells (10-30% between 2-6 hr) and NHEK (23-30% between 0.5-3 hr). Depletion of cellular GSH by buthionine sulfoximine (1 mM), a specific GSH biosynthesis inhibitor, also increased $[Ca^{2+}]_i$ (88% at 1 hr) in NHEK, suggesting that GSH depletion may lead to increased $[Ca^{2+}]_i$. Calcium, localized cytochemically with antimony, accumulated in increased amounts around mitochondria and endoplasmic reticula, in the cytosol, and in particular in the euchromatin regions of the nucleus beginning at 6 hr after 0.3 mM SM exposure of NG108-15 cells. Cell membrane integrity (NHEK) examined with the fluorescent membrane probe calcein AM was unaffected through 6 hr following 1 mM SM exposure; and cell viability (NG108-15 cells) measured by trypan blue exclusion was >80% of control through 9 hr following 0.3 mM SM exposure. Since $[Ca^{2+}]_i$ increase precedes cell membrane damage or viability loss due to SM, these data support a mechanism of SM toxicity that involves an early $[Ca^{2+}]_i$ increase, which could initiate a cascade of toxic events, e.g., phospholipase (PLA_2) activation, arachidonic acid release, and membrane fluidity decrease as demonstrated previously (Ray et al., The Pharmacologist, 34(3):174, 1992).

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INTRODUCTION

Alkylating agents such as sulfur mustard (SM, bis-(2-chloroethyl)sulfide) are strong electrophiles that can readily react with a host of cellular molecules including glutathione (GSH), which is critical for the functioning of Ca^{2+}-ATPases (Ca^{2+}-pumps) (Bellomo et al., 1983). The plasma membrane Ca^{2+}-pump is responsible for Ca^{2+} efflux, whereas the mitochondrial and the endoplasmic reticulum Ca^{2+}-pumps are responsible for buffering of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Orrenius, 1985; Orrenius and Nicotera, 1987). Depletion of cellular GSH by SM may therefore increase $[Ca^{2+}]_i$, resulting in Ca^{2+}-mediated toxicity. High $[Ca^{2+}]_i$ can cause activation of phospholipases, e.g., phospholipase A_2 (PLA_2) and proteases (Orrenius, 1985). Enhanced PLA_2 activity may cause arachidonic acid (AA) release from cell membrane leading to a decreased membrane fluidity and consequent abnormality in membrane function. In this report, we present the results of our study on SM-induced intracellular Ca^{2+} changes in in vitro cell culture models.

MATERIALS

Sulfur mustard was obtained from the Chemical Research Development and Engineering Center, Aberdeen Proving Ground, MD, and subsequently analyzed and supplied (98% pure) to us by the Analytical Chemistry Branch of this Institute. Glutathione, glutathione disulfide (GSSG), NADPH, 5,5'-dithiobis(2-nitrobenzoic acid (DTNB), and 5-sulfosalicylic acid were purchased from Sigma Chemical Company (St. Louis, MO). Fluo-3 AM and calcein AM were purchased from Molecular Probes (Eugene, OR), and anhydrous dimethyl sulfoxide (DMSO) was purchased from Aldrich (Milwaukee, WI). Normal human epidermal keratinocytes (NHEK) and keratinocyte growth medium (KGM) were purchased from Clonetics corporation (San Diego, CA). Powdered Dulbecco's modified Eagle's minimal essential medium with L-glutamine and high glucose (DMEM), hypoxanthine, aminopterin, thymidine, and trypan blue were products of GIBCO Laboratories (Grand Island, NY). Fetal bovine serum was obtained from Hyclone Laboratories (Logan, UT). The NG108-15 cell line was a gift from Dr. M. Nirenberg of the National Institutes of Health, Bethesda, MD. All other chemicals were of analytical reagent grade.

METHODS

Cell culture

Cells were grown in 150 cm^2 plastic tissue culture flasks containing 50 ml growth medium (pH 7.2-7.4). Keratinocytes were grown in KGM supplemented with bovine pituitary extract. NG108-15 cells were grown in 95% (v/v) DMEM supplemented with 5% (v/v) fetal bovine serum, 100 μM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine. The ambient conditions of incubation were a humidified atmosphere of 5% CO_2/95% air for NHEK, and 10% CO_2/90% air for

NG108-15 cells, and a temperature of 37°C. Keratinocytes were subcultured until passage 4 or 5 using 50-75% confluent cells, whereas NG108-15 cells were subcultured until passage 30 using fully confluent cells. All cultures used in experiments were highly confluent.

Treatment of cells with SM

Cells were treated with diluted stock SM in KGM, DMEM, or isosmotic (340 mOsmoles/L) HEPES buffered salt solution (HBSS: NaCl 112 mM, KCl 5.4 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 0.8 mM, NaH_2PO_4 0.9 mM, $NaHCO_3$ 1 mM, D-glucose 10 mM, HEPES 25 mM, pH 7.4) for specified concentration and time. Sulfur mustard is very sparingly soluble in water and hydrolyzes rapidly ($t_{1/2}$ < 10 min) in aqueous medium. Therefore, a special procedure was used for exposing cells to SM in aqueous medium using a unique formulation developed by Broomfield and Gross (1989). Appropriate dilutions were made for exposure of cells by adding aliquots of stock SM solution to cells suspended in specified medium. All operations during the first hour of exposure of cells to SM were conducted inside a total exhaust laminar flow chemical and biological safety cabinet to protect against personnel exposure.

Glutathione assay

Cell pellets were suspended in 5% 5-sulfosalicyclic acid to obtain a 10^7 cell/ml suspension, which was sonicated on ice using a Branson sonicator set in pulse mode at 80% of maximum power. The suspension was sonified for six 15-sec bursts separated by 30-sec intervals. Cell lysis was essentially complete as verified under a microscope. Samples were vortexed and kept on ice for 30 min for complete extraction, and then centrifuged at 12,000 x g for 5 min. Supernatants were withdrawn and assayed for glutathione (GSH + GSSG) using the glutathione reductase cycling assay (Griffith, 1980). Absorbance was measured by a Beckman DU-70 spectrophotometer.

Determination of intracellular free calcium level

Intracellular free Ca^{2+} level was determined fluorometrically using the Ca^{2+}-specific fluorescent probe Fluo-3 AM following the principle described by Tsien et al. (1982). The cell membrane permeant acetoxymethyl ester of Fluo-3 (Fluo-3 AM) is hydrolyzed inside the cell to generate the membrane impermeant free acid Fluo-3, which then combines with free Ca^{2+} to produce a fluorescence signal proportional to the intracellular free Ca^{2+} concentration. Neither Fluo-3 AM nor Fluo-3 itself is fluorescent. Assays were done using either washed confluent monolayer NHEK in 24-well plates or suspensions of washed NG108-15 cells (5 x 10^6 cells per assay) in HBSS. Cells were incubated at room temperature in HBSS containing 25 μM Fluo-3 AM with or without 0.3 mM SM. At specified times, monolayer NHEK or aliquots of NG108-15 cell suspension were washed once using HBSS by either aspiration (for monolayer NHEK) or centrifugation (for NG108-15 cells), and then fluorescence was read at an excitation/emission wave length setting of 485 nm/530 nm in a Millipore

Cytofluor 2300 fluorescence measurement system. Intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$ was calculated using the equation $[Ca^{2+}]_i = 450\ nM\ (F - F_{min})/F_{max} - F)$, where 450 nM is the dissociation constant for Fluo-3, F is the fluorescence signal in situ of cells with the unknown $[Ca^{2+}]_i$, F_{min} and F_{max} are the minimum and maximum Ca^{2+} fluorescence respectively in the test sample. Values of F_{min} and F_{max} were obtained by either adding EGTA (2 mM) prior to Fluo-3 AM or dissolving Fluo-3 loaded cells in a detergent solution (1% Triton X100) respectively. Background fluorescence in 0.3 ml HBSS alone or with equivalent cells in wells was subtracted in calculation of results.

Calcium histochemistry

Untreated and 0.3 mM SM-treated cells were fixed at selected post exposure times at 4°C for 1-3 hours in a combined aldehyde fixative of 1.6% formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.45 and 190 mOsmoles/L) containing 50 mM calcium chloride. Cells were then washed in buffer and incubated in a medium of 2% potassium pyroantimonate and 1% osmium tetroxide for 90 min at 20°C in the dark. Following incubation, cells were washed and placed in a secondary medium of 0.5% tannic acid for 15 min, rinsed in 1% sodium sulfate for one min, dehydrated in graded ethanols and embedded in epoxy resin. Ultrathin sections were analyzed without counterstaining to maximize visualization of calcium-pyroantimonate precipitates.

Determination of membrane integrity

Membrane integrity or cell viability was determined by two different methods: (a) trypan blue dye exclusion assay based on the viable cell membrane's ability to exclude the dye taken up by the cell, and (b) calcein fluorescence assay based on the viable cell membrane's ability to hydrolyze the fluorescent probe calcein AM taken up by cells. Loss of membrane integrity is indicated by a reduction of dye exclusion or calcein fluorescence. For trypan blue exclusion assay, a cell suspension (approximately 10^6 cells/ml) was incubated at room temperature for 5 min in HBSS with 0.1% trypan blue. An aliquot of the suspension was then counted in a hemacytometer for the proportions of stained (dead) and clear (living) cells. For calcein fluorescence assay, cells were incubated at room temperature for 30 min in HBSS with 5 μM calcein AM. Fluorescence was read at an excitation/emission wave length setting of 485 nm/530 nm in a Millipore Cytofluor 2300 fluorescence measurement system. Background fluorescence in HBSS alone or with equivalent cells in wells was subtracted in calculation of results.

RESULTS

The effect of SM on intracellular Ca^{2+} level was studied in vitro using NHEK and NG108-15 cells. Following 0.3 mM SM exposure, GSH level decreased 20-34% between 1-6 hr in NG108-15 cells (Table 1). SM increased $[Ca^{2+}]_i$, measured using the Ca^{2+}-specific fluorescent probe Fluo-3 AM, in both NG108-15 cells (10-30% between 2-6 hr) as shown in Fig. 1, and NHEK (23-30% between 0.5-3 hr) as shown in Fig. 2. Depletion of cellular GSH by buthionine sulfoximine (1 mM), a specific GSH biosynthesis inhibitor, also increased $[Ca^{2+}]_i$ in NHEK (Table 2). Calcium, localized cytochemically with antimony, accumulated in increased amounts around mitochondria and endoplasmic reticula, in the cytosol, and in particular in the euchromatin regions of the nucleus beginning at 6 hr after 0.3 mM SM exposure (Fig. 3). Cell membrane integrity examined with the fluorescent membrane probe calcein AM was unaffected through 6 hr (Fig. 4), and cell viability measured by trypan blue exclusion was >80% of control through 9 hr post 0.3 mM SM treatment (Fig. 5).

Table 1

Effect of Sulfur Mustard on Glutathione Level in NG108-15 Cells

Time	GSH content (nmoles/10^6 cells)*		
(hr)	Control	SM	Mean % decrease due to SM
1	12.51	10.01	20
6	23.44	15.41	34

*Each value is the mean from two separate experiments in which assays were done in quadruplicates. GSH content was determined as described in Methods at 1 and 6 hr following incubation of cells in HBSS (control) or in HBSS containing 0.3 mM SM.

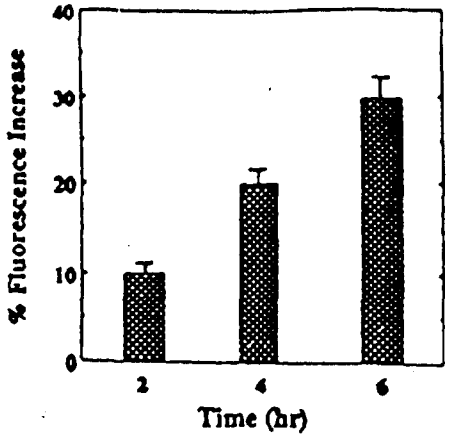

Fig. 1. SM-induced increase in $[Ca^{2+}]_i$ in NG108-15 cells. The effect of 0.3 mM SM exposure on $[Ca^{2+}]_i$ was studied by using the Ca^{2+}-specific fluorescent probe Fluo-3 AM as described in Methods. When cells were exposed to 0.3 mM SM in HBSS in the presence of 25 μM Fluo-3 AM, a time-dependent increase (10-30% between 2-6 hr) in the fluorescence due to the formation of Ca^{2+}-Fluo-3 complex was observed indicating that SM caused an increase in $[Ca^{2+}]_i$. Sulfur mustard increased $[Ca^{2+}]_i$ compared to respective controls at 4 hr and 6 hr ($p < 0.05$, $N = 3-6$).

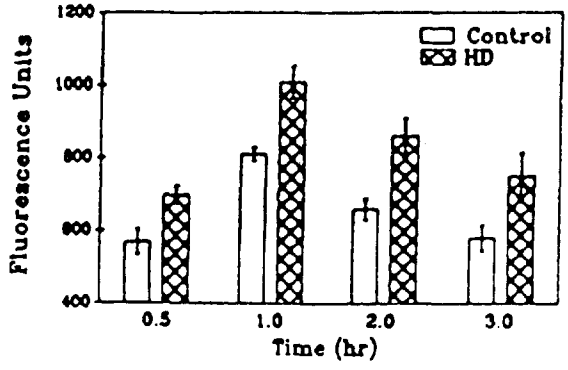

Fig. 2. SM-induced increase in $[Ca^{2+}]_i$ in NHEK. The effect of 0.3 mM SM exposure on $[Ca^{2+}]_i$ was studied by using the Ca^{2+}-specific fluorescent probe Fluo-3 AM as described in Methods. When cells were exposed to 0.3 mM SM in HBSS in the presence of 25 μM Fluo-3 AM, an increase in the fluorescence due to the formation of Ca^{2+}-Fluo-3 complex was observed indicating that SM caused an increase in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ in SM-treated cells was 23-30% higher than corresponding controls between 0.5-3 hr ($p < 0.05$, $N = 3-6$).

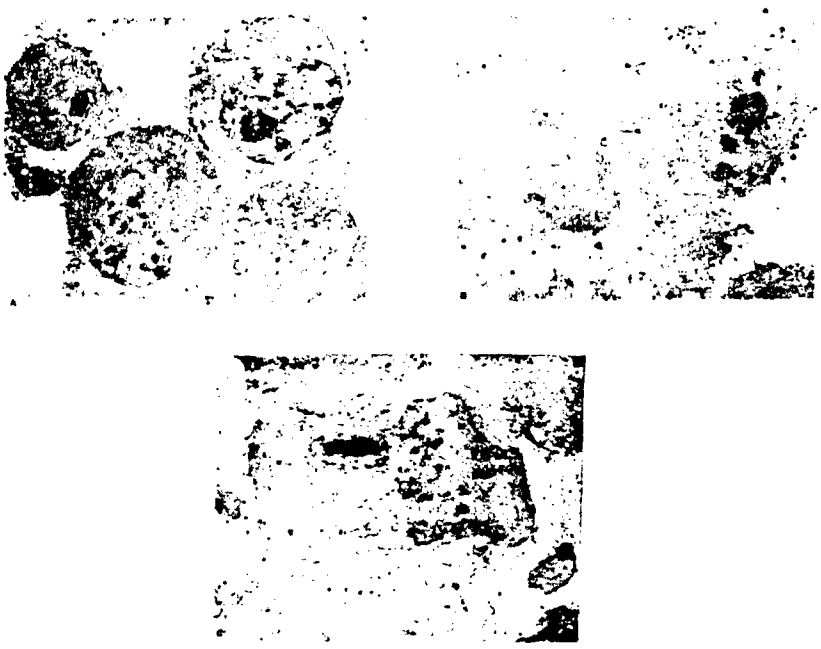

Fig. 3. Cytochemical localization of SM-induced change in intracellular calcium. Cellular calcium was depicted by calcium-antimony precipitates as described in Methods. A. Up to 3 hours following HD exposure, calcium (arrows) was localized largely to the plasma membrane, microvilli (Mv) and to extracellular domains. Magnification 7500X. B. Six hours post HD exposure, calcium was localized to the limiting membrane of swollen endoplasmic reticulum (Er), to euchromatin areas of the nucleus (N) and to areas of the cytosol and plasma membrane (arrows). Magnification 7500X. C. At 12 hours calcium was localized increasingly to euchromatin of the nucleus (arrows), to mitochondria (M) and to progressively swollen endoplasmic reticulum. Magnification 9000X

Table 2

Effect of BSO on $[Ca^{2+}]_i$ in NHEK

Experimental condition	Fluorescence Units* Mean ± SEM
Control	1874 ± 103
1mM BSO	3590 ± 103

*Each value is the mean from 5 determinations. Monolayer NHEK cultures were first incubated in either HBSS (control) or in HBSS containing 1 mM BSO for 1 hr. These cells were washed in HBSS and then $[Ca^{2+}]_i$ was determined as described in Methods. Treatment of cells with 1 mM BSO increased $[Ca^{2+}]_i$ by 88% compared to untreated control cells ($p < 0.05$).

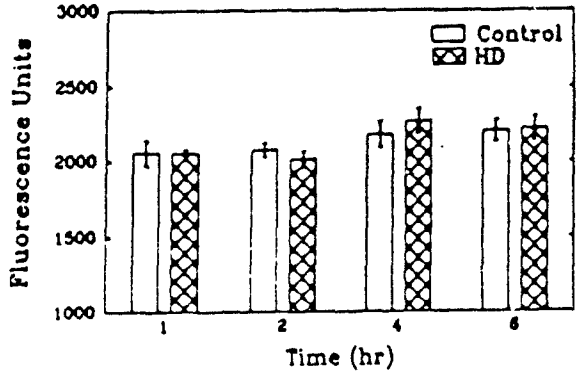

Fig. 4. Effect of 1 mM SM on membrane integrity in NHEK. The effect of 1 mM SM exposure on cell membrane integrity was studied by using the fluorescent membrane probe calcein AM as described in Methods. Confluent monolayer cells were exposed to 1 mM SM in HBSS for specified times. During the last 30 min of these incubation times, SM solutions were replaced by 5 μM calcein AM solution in HBSS. Washed cells were read for calcein fluorescence. There was no change in fluorescence during 1-6 hr following 1 mM SM exposure, suggesting no effect of SM on membrane integrity up to 6 hr.

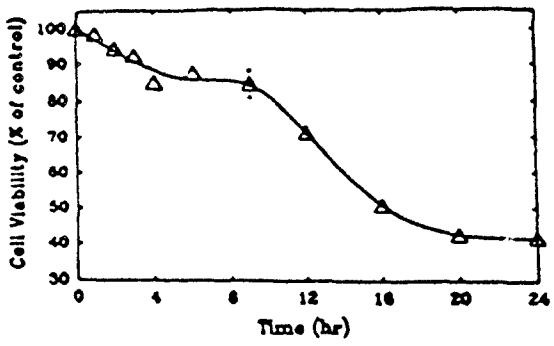

Fig. 5. The effect 0.3 mM SM on NG108-15 cell viability. Cell viability was determined by the trypan blue exclusion assay as described in Methods. Results are % of untreated control (≥ 98% viable). Each value is the mean ± SEM of 5-15 determinations. SEM bars are not visible at some time points because those are smaller than the symbol size. Following SM exposure, the apparent small decrease (5-15%) in cell viability between 2-9 hr was not statistically significant. However, cell viability decreased progressively between 12-24 hr to about 40% of control in 20-24 hr.

DISCUSSION

The results of this study demonstrate that in in vitro cell cultures SM increases $[Ca^{2+}]_i$. The observations that (a) SM decreases cellular GSH level and (b) inhibition of cellular GSH biosynthesis by BSO also increases $[Ca^{2+}]_i$ suggest that one of the mechanisms of SM-induced $[Ca^{2+}]_i$ increase may be via GSH depletion. The observation that GSH biosynthesis inhibition by BSO leads to an increase in $[Ca^{2+}]_i$ is interesting, and whether this BSO-induced $[Ca^{2+}]_i$ increase can cause any cellular toxicity is being investigated in our laboratory at present. Exposure of cells to 0.3 mM SM caused a modest (20-30%) increase in $[Ca^{2+}]_i$. However, EM studies showed that this increased Ca^{2+} was localized in certain areas of the cell, e.g., around swollen endoplasmic reticula and mitochondria, in the cytosol and plasma membrane, and particularly in the euchromatin regions of the nucleus. It is possible that such localization of Ca^{2+} at specific areas of the cell could result in local high Ca^{2+} concentrations at those areas and thus initiate Ca^{2+}-mediated toxic events such as activation of phospholipases, proteases, and endonucleases (Orrenius, 1985; Orrenius and Nicotera, 1987). Previous reports from this laboratory suggested SM-induced PLA_2 activation leading to arachidonic acid release from cell membrane and consequent decrease in membrane fluidity (Ray et al., 1992). SM-induced $[Ca^{2+}]_i$ increase occurred within 6 hr, when there was no evidence of any membrane damage as determined by trypan blue exclusion

assay or calcein fluorescence assay. These results suggest that following SM exposure, increase in $[Ca^{2+}]_i$ is an early event which may in part be related to a cascade of toxic mechanisms responsible for SM-induced pathology such as vesication. The knowledge obtained from this study may be useful in developing methods of prevention and therapy of SM toxicity.

ACKNOWLEDGMENTS

We thank Dr. J. J. Yourick for help in the statistical analysis of the data. We acknowledge the contribution of Mr. K. R. Mills and Ms. T. A. Hamilton in conducting the electron microscopic studies.

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